Chronic helminth infection perturbs the gut-brain axis, promotes neuropathology and alters behaviour

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Short title: Helminths alter microbiome and behaviour

summary: Helminth infections during childhood are associated with impaired learning ability. The present study demonstrates a potential mechanism by which worms influence cognitive function, via acute changes in the gut microbiome and lasting evidence of pathology in the brain tissue.

ABSTRACT

Helminth infections in children are associated with impaired cognitive development, however the biological mechanisms for this remain unclear. Using a murine model of gastrointestinal helminth infection, we demonstrate that early-life exposure to helminths promotes local and systemic inflammatory responses and transient changes in the gastrointestinal microbiome. Behavioural and cognitive analyses performed 9-months post-infection revealed deficits in spatial recognition memory and an anxiety-like behavioural phenotype in worm-infected mice, which was associated with neuropathology and increased microglial activation within the brain. This study demonstrates a previously unrecognised mechanism through which helminth infections may influence cognitive function, via perturbations in the gut-immune-brain axis.

KEYWORDS: helminth, microbiome, cognition, inflammation

BACKGROUND

Soil-transmitted helminth (STH) infections, such as *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms, cause significant morbidity, particularly in children, including anaemia, reduced growth [1], impaired cognitive function and reduced educational advancement [2]. Multiple studies have looked at the effects of STHs on cognitive function, with some conflicting evidence regarding their causal role that likely reflects the complexity in human population-based studies [3, 4]. Hence, more carefully controlled studies in human populations and animal models are required. The mechanisms by which STHs influence cognitive development also remain unclear, and are likely to involve both direct effects of nutritional deficits on the brain and indirect effects of pathophysiological events occurring in the gut environment. Recent research has highlighted the crucial role for the intestinal microbiome in regulating central nervous system (CNS) development, especially synaptogenesis and myelination [5]. Disruption of gut microbial homeostasis (i.e. dysbiosis) can affect emotional behaviour and related brain systems, leading to a range of mental health disorders including autism spectrum disorder, anxiety, depression and chronic pain [6]. There is a bidirectional functional communication between gut microbiota, the gastrointestinal tract and the CNS; these relationships have been recognised as the microbiota-gut-brain axis, which operates through a variety of physiological mechanisms, including neural, hormonal and immunological pathways [7].

Recent evidence from human and animal studies indicates that helminth infections can influence the gut microbiota [8]. These interactions may be beneficial for some inflammatory conditions [8], but may be detrimental if they occur during a critical window of childhood development and if worm burdens are high. In a recent study we hypothesized that helminth-induced changes in the gut microbiome are associated with intellectual development at a number of critical time windows, including prenatal, post-natal, during adolescence and into adulthood [9]. In the present study, we extend on these findings by employing a murine model of helminth infection. We describe a

previously unrecognised pathway by which helminths may negatively impact brain structure, behaviour and cognitive function, by disturbing the homeostasis of the gut microbiota and the immune system.

METHODS

Study design

Male wild type C57BL/6 mice were maintained at James Cook University (JCU). All protocols were approved by the JCU Animal Ethics Committee. *Trichuris muris* was maintained in genetically susceptible mice and excretory/secretory (ES) antigens were derived from adult worms. At 8-weeks of age, groups of 12 mice received either 30 *T. muris* eggs, 6 *T. muris* eggs or saline placebo (naïve) by oral gavage. Researchers performing behavioural, microbiome and histological analyses were blinded as to the treatment group. Blood was collected by cardiac puncture or submandibular bleeding and serum was stored at -80°C. Colon tissue (1 cm sections) were sliced longitudinally and cultured overnight at 37°C (organ culture). *Trichuris* ES-specific cytokine responses were assessed by restimulation of mesenteric lymph node (mLN) cells with 50 µg/mL ES. Serum and culture supernatants were assessed for cytokine levels using ELISA (eBioscience).

DNA isolation, 16S rRNA gene amplicon sequencing and analysis

Faecal samples were collected and pooled from each cage of 6 mice and frozen at -80°C before analysis. Samples were put into a 5% potassium dichromate solution prior to DNA extraction using the PowerSoil® DNA extraction kit (MoBio). Illumina paired-end sequencing (MiSeq) was performed on the V4 region of the 16S ribosomal RNA gene as amplified using primers based on the Earth Microbiome Project primers 515F and 806R. Raw data were quality filtered and trimmed using trimmomatic version 0.32 (with parameters PE, leading 15, trailing 15, minlen 180. Paired reads were then assembled with PEAR allowing a minimum overlap of 20bp. Individual read pairs were converted to fasta and qual files. Sequence data analysis was done in QIIME version 1.9.1.

Operational taxonomic units (OTUs) were assigned from sequence reads using a closed-reference OTU picking protocol against the Greengenes reference database (version 13_8) clustered at 97% similarity threshold with uclust. Taxonomy was assigned with the RDP classifier accepting the Greengenes taxonomy string of the best matching Greengenes sequence. Statistical analyses including were done in R, including functions from the vegan and indicspecies packages. Shifts in bacterial community composition were visualized with non-metric multidimensional scaling (nMDS, function metaMDS in vegan package) and the effects of sampling time and infection treatment were tested with PERMANOVA (function adonis in vegan package). OTUs that were significantly overrepresented ($p>0.05$, adjusted for multiple testing) in either infection treatment were identified with the function signassoc (indicspecies package). Only these "indicator OTUs" were displayed in the nMDS ordination.

Behavioural assessments

Animal behaviour was tested at ten months of age using tests and procedures described in detail in Kraeuter *et al* 2018 [10]. In brief, to assess learned helplessness/depression-like behaviour the **forced swim test** was used; this test was conducted in a 2000 ml beaker filled with 1400ml of water. Mice were placed into the water and the activity (mobility, immobility, latency to immobility) was video-recorded for 6 minutes. The **open field test** was used to assess psychomotor activity and "emotionality". The test mouse was placed into the middle of the apparatus (420 x 420 x 420 mm; 205 x 205 mm centre zone) for 15 minutes. Behaviour in the open field was recorded by a video camera above the arena. A **Spatial reference memory Y-maze test** was used to assess spatial reference memory. During the Training session one arm of the Y-maze was closed. The animal was placed into the start arm facing the centre and was allowed to freely explore the "start" and the "other" arm for fifteen minutes during the acquisition phase. One hour later, during the Testing session, the animal was placed back to the apparatus and the barrier was removed to allow the animal to explore the entire arena (start arm, "other arm", novel arm) for five minutes.

Behaviour was recorded by a video camera placed above the arena. Video recordings were analysed using TopScan Light (Cleaver Sys Inc., Reston, Virginia, USA).

Pathophysiological studies

Histological studies were performed on tissue sections from 4 mice from each group. Mice were perfused with 4% paraformaldehyde and brain tissue was fixed overnight in paraformaldehyde and transferred to 30% sucrose solution prior to tissue sectioning and immunohistochemical staining. Microglial activation was assessed by staining with a rabbit polyclonal IbaI antibody (Wako Cat. No. 019-19741). The degree of immunolabelling across serial sections was ascertained as a measure of activation and degeneration. Neuronal degeneration was assessed using highly sensitive Fluorojade C staining (AG325 Merck) according to the manufacturer's instructions.

Statistics

Statistical analysis was performed using the SPSS version 23 software package (IBM SPSS Statistics). $p < 0.05$ will be considered significant throughout all data. All data are depicted as mean ± Standard error of mean (SEM).

RESULTS AND DISCUSSION

As expected, infection of mice with 30 *T. muris* eggs resulted in inflammatory pathology in the large intestine after 1 month (**Fig. 1A**), and antigen-specific IFNγ responses in mLN (**Fig. 1B**). Our primary study involved examining the effect of different burdens of helminth infections on systemic inflammation, the microbiome and behaviour (**Fig. 1C**). Hence, we treated groups of 12 mice with either 30 *T. muris*, 6 *T. muris* or saline placebo (naïve) by gavage. *Trichuris* infection resulted in lower mouse weight at 4 months (Naive: 36.4±2.4 g, 6 *Trichuris*: 35.1±2.9 g and 30 *Trichuris*: 34.4±1.7 g, mean±SD), however these differences were not statistically significant when using a Mann-Whitney t-test. Analysis of systemic inflammatory responses revealed significant elevations in

the pro-inflammatory cytokine IFNγ in the serum at 1 month, which returned to naïve levels by 4 months. (**Fig. 1D**). Levels of other pro-inflammatory cytokines IL-1β and IL-17A were not elevated following infection (**Fig. 1E-F**).

These acute changes in inflammatory parameters were accompanied by changes in the composition of the faecal microbiome. Faecal pellets taken at 1, 4 and 9 months p.i., as well as naïve mice were subjected to 16S rRNA sequencing. Our results revealed a *Trichuris*-dependent shift in microbiota composition one month p.i. in infected mice compared to naïve mice (**Fig. 1G**). Four and nine months p.i. the microbiota became increasingly similar to the microbiota of naive mice (**Fig. 1G**). Infection dose explained 29% of variation in microbiome community composition (PERMANOVA p=0.001) whereas sampling time explained 18% (PERMANOVA p=0.04). We observed a generally higher relative abundance of *Bacteroidetes* and lower abundance of *Firmicutes* in 1-4 month infected mice compared to naïve mice (**Fig. 1H**). This was confirmed by indicator species analysis, which associated many OTUs of *Firmicutes* (*Clostridiales*) with a healthy murine gut microbiota, while *Bacteroidales* were associated with infected mice. The analysis revealed that OTUs affiliated with *Enterobacteriales* are indicator taxa for the severity of infection during the acute phase, consistent with previous reports [11, 12], which is indicative of a dysbiotic "inflammatory" microbiota.

At 8 months post-infection we subjected mice to a range of validated and well-characterised behavioural tests, which were performed by an investigator blinded to the treatment. A forced swim test revealed that mice that received 30 *Trichuris* displayed reduced mobility (**Fig 2A**) and increased immobility (**Fig 2B**) in the water, suggestive of depression-like behaviour. The open field test demonstrated that 30 *Trichuris* treated mice had reduced total distance travelled (**Fig. 2C**) and lower number of entries into centre area (**Fig. 2D**) compared to controls, indicating a decreased psychomotor activity and a potential depression/anxiety like-behavioural phenotype. The reference

Y-maze test revealed that while naïve control and 6 *Trichuris* infected mice spent more time in the novel arm of the maze than in the other arm, which indicates good spatial memory, 30 *Trichuris* infected mice spent approximately the same amount of time in each arm (**Fig. 2E**), consistent with impaired spatial reference memory. 30 *Trichuris* treated mice did not display significantly decreased activity as defined by total distance travelled in the Y-maze during testing (**Fig. 2F**), suggesting that impaired spatial memory was not due to motor impairment. Together these data demonstrate that chronic, higher-intensity helminth infection may be associated with significant changes in behaviour, chiefly a depression-like phenotype and impaired memory. Critically, histopathological analysis of brain tissue sections (amygdala and adjacent cortical region) revealed clear increases in presence of Flurojade C-positive degenerating neurons in 30 *Trichuris*-treated mice compared to naïve (**Fig. 2G)**. Furthermore, increased Iba1 staining within cortical tissue was observed, indicating increased microglial activation (**Fig. 2H**). Analyses of brain pathology from mice treated with 6 *Trichuris* did not reveal obvious differences in Iba1 staining compared to naïve control mice (data not shown). These data suggest that exposure to heavier helminth burdens earlier in life were more strongly associated with persistent pathological changes in the brain, potentially accounting for the changes in behaviour. To our knowledge, this is the first report of neuropathology associated with a helminth infection. Further studies are required to assess potential causal relationships of the worm infection, or the relative importance of the changes in the microbiome and inflammatory responses. Regardless, our findings fit in with the broader paradigm shift in neurobiology and neurodegeneration where increasingly the gut microbiome (and perhaps the macro-biome, i.e. worms) and interrelated factors that disturb normal homeostasis such as stress, diet, malnutrition and infections, are a major pathological trigger that can cause similar phenotypes in animal models of neurological disease [13]. Several observational studies reported that exposure to STH infections during pregnancy or in school aged children can impair the efficiency of cognitive processes including memory, learning, motor development, verbal fluency and non-verbal intelligence [14, 15]. Our study demonstrates that helminth infections may affect the

gut-brain axis, altering behaviour and causing neuropathology. The helminth-gut microbiota-CNS hypothesis needs support from further animal model and human epidemiological studies that would more definitively determine the potential causal relationship between helminth infections in early life (pre-natal exposure and <3 years of age), impaired cognitive development and the microbiome. This could involve the undertaking of de-worming studies using the animal model described herein, to test whether differential timings of anthelminthic treatment could reverse this behavioural phenotype. Such experiments could inform the most appropriate age to implement community-deworming procedures in humans (currently aimed at school-age children), which could limit the development of CNS and behavioural disorders in these at risk populations.

FOOTNOTES

1. Conflicting interests

The authors declare that they have no conflicting interests.

2. Financial support

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5. FIGURE LEGENDS

Figure 1. Chronic helminth infection elicits intestinal pathology, systemic inflammation and alterations in gut microbiome composition and abundance. Wild type C57BL/6 mice were infected with 30 embryonated *Trichuris muris* eggs, or were uninfected (naïve). (**A**) Representative photomicrographs of caecal histopathology at 1 month post-infection (p.i.). (**B**) Levels of IFNγ in colon organ culture supernatants or *Trichuris* antigen-restimulated mesenteric lymph node supernatants at 1 month p.i. **(C)** Main study examining effect of infection with 6 *Trichuris* eggs or 30 *Trichuris* eggs on inflammatory, microbiota and behavioural parameters**.** Serum levels of (**D**) IFNγ, (**E**) IL-17A and (**F**) IL-1β at various time points p.i. Data are expressed as mean + SEM, * denotes $p<0.05$ compared to naïve control. **(G)** The composition of mouse gut microbiome visualized using nMDS. Each point refers to a pooled fecal sample consisting of fecal pellets from two cages of 6 mice/group at each time point. Points are color-coded according to infection treatment and shaded according to sampling time. Ellipses have been drawn around healthy and infected treatment groups to aid interpretation **(H)** OTUs that contribute significantly to the differences in microbiome composition in response to infection treatment (indicator OTUs). Each cross-refers to one OTU, color-coded by taxonomic assignment (bacterial order level). OTUs within or close to a treatment group ellipse are overrepresented in that treatment group.

Figure 2. Chronic *Trichuris* infection induces a depression-like behaviour phenotype, reduced spatial memory and neuropathology. Behavioural tests were performed at 8 months post-infection. Percent (**A**) mobility and (**B**) immobility in a forced swim test. (**C**) Total distance travelled (m) and (**D)** number of centre entries in the open field test. (**E**) Relative amount of time spent in 'Start' (S) arm, 'Novel' (N) arm or 'Other' (O) arm assessed using the spatial reference memory Y-maze test. (**F**) Total distance travelled in the reference Y-maze test. Histopathological analysis of brain pathology was performed at necropsy (9 months p.i.). (**G**) Photomicrographs of

Fluorojade C-staining in amygdala and adjacent cortical region (the cortex-amygdala transition zone) tissue sections from naïve control or high-dose *Trichuris* infected mice. (**H**) Photomicrographs of IBa1 staining in cortical tissue. Histological studies were performed on tissue sections from 4 mice from each group and representative images are shown. Data are expressed as mean + SEM, * denotes $p<0.05$.

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Fig. 1

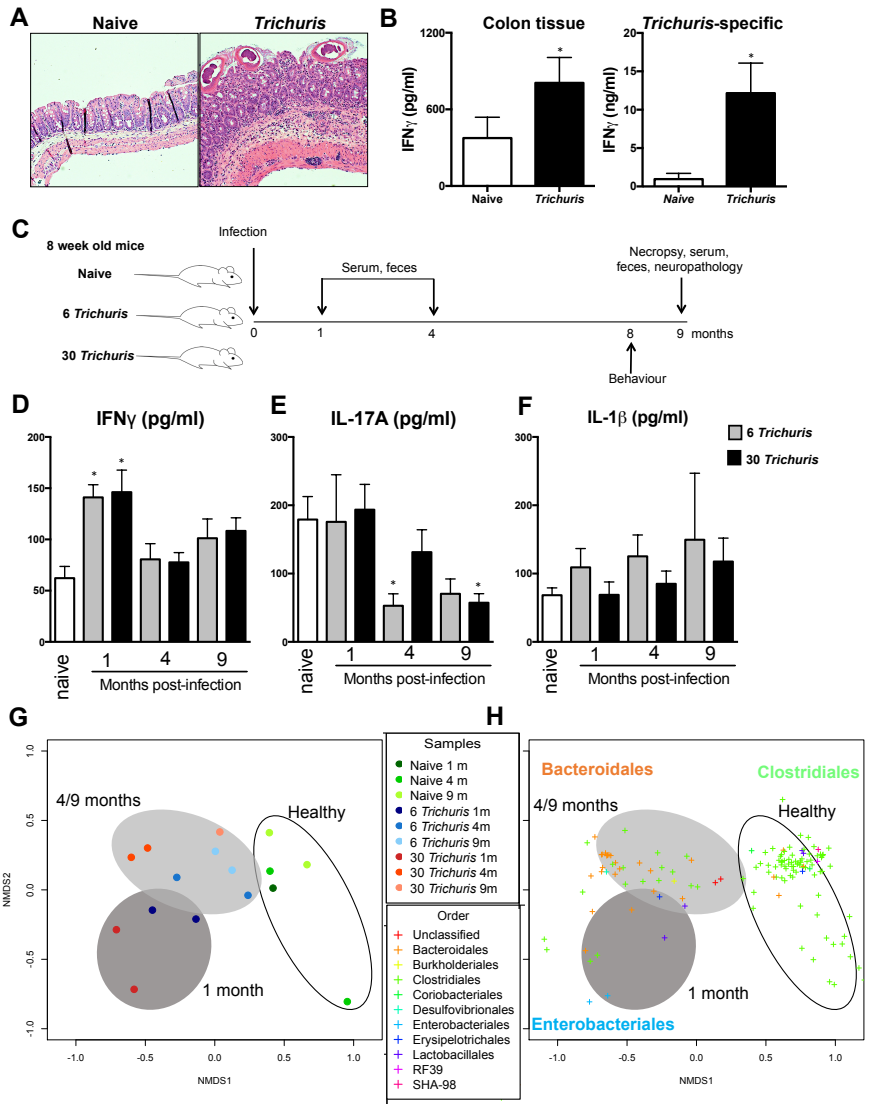

Fig. 2